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25-Hydroxyvitamin D_3 is an active hormone in human primary prostatic stromal cells

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ABSTRACT

According to the present paradigm, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] is a biologically active hormone; whereas 25-hydroxyvitamin D_3 ($25OHD_3$) is regarded as a prohormone activated through the action of 25-hydroxyvitamin D_3 1α-hydroxylase (1α-hydroxylase). Although the role of vitamin D_3 in the regulation of growth and differentiation of prostatic epithelial cells has been well studied, its action and metabolism in prostatic stroma are still largely unknown. We investigated the effects of $25OHD_3$ and 1α,25-$(OH)_2D_3$ on two human stromal primary cultures termed P29SN and P32S. In a cell proliferation assay, $25OHD_3$ was found at physiological concentrations of 100–250 nM to inhibit the growth of both primary cultures, whereas 1α,25-$(OH)_2D_3$ at a pharmacological concentration of 10 nM exhibited the growth-inhibitory effects on P29SN cells but not on P32S cells. Quantitative real-time RT-PCR analysis revealed that both $25OHD_3$ and 1α,25-$(OH)_2D_3$ induced 25-hydroxyvitamin D_3 24-hydroxylase (24-hydroxylase) mRNA in a dose- and time-dependent manner. By inhibiting 1α-hydroxylase and/or 24-hydroxylase enzyme activities, the induction of 24-hydroxylase mRNA by 250 nM $25OHD_3$ was clearly enhanced, suggesting that 1α-hydroxylation is not a prerequisite for the hormonal activity of $25OHD_3$. Altogether our results suggest that $25OHD_3$ at a high but physiological concentration acts as an active hormone with respect to vitamin D_3 responsive gene regulation and suppression of cell proliferation.

Key words: fibroblast • prostate cancer • cell proliferation • 25-hydroxyvitamin D_3 24-hydroxylase • 25-hydroxyvitamin D_3 1α-hydroxylase

Several decades ago, the elucidation of the role of 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] in calcium and phosphorus homeostasis caused researchers to focus on the mechanism of 1α,25-$(OH)_2D_3$ action, which in turn led to the discovery of vitamin D receptor (VDR). The expression of VDR was observed in other tissues not previously considered targets, such as skin (1), ovary (2), and prostate, including several human prostate cancer cell lines (3); primary cultures of prostatic epithelial and fibroblastic cells from normal, benign hyperplastic, and malignant tissues (4); and the normal prostate tissue (5). It was found

that 1α,25-$(OH)_2D_3$ modulates cell proliferation, differentiation, cancer invasion and angiogenesis (6–9). Accordingly, 1α,25-$(OH)_2D_3$ is suggested to be a potential preventive and therapeutic agent against prostate cancer. However, the major side effects of 1α,25-$(OH)_2D_3$ are hypercalcemia and hypercalciuria, which limit its therapeutic use (10–12).

The major circulating metabolite 25OHD_3 is activated by 25-hydroxyvitamin D_3 1α-hydroxylase (1α-hydroxylase, CYP27B1) in kidney (13–16). The active hormone, 1α,25-$(OH)_2D_3$, as well as 25OHD_3, are inactivated by 25-hydroxyvitamin D_3 24-hydroxylase (24-hydroxylase, CYP24) in kidney (17, 18) and in the other vitamin D_3 target tissues (19). 24-Hydroxylase is a multicatalytic enzyme that catalyzes the side-chain oxidation of 25OHD_3 metabolites, notably 25OHD_3 and 1α,25-$(OH)_2D_3$. The induction of 24-hydroxylase gene expression by 1α,25-$(OH)_2D_3$ has been used frequently as an indicator of transcriptional activity of vitamin D_3 metabolites because there are two vitamin D-responsive elements in CYP24 gene (20). Under normal physiological conditions, the inhibition of 1α-hydroxylase expression and the marked induction of 24-hydroxylase expression by 1α,25-$(OH)_2D_3$ in kidney provide a strict control of the concentration of circulating 1α,25-$(OH)_2D_3$ (19). This regulation is partially mediated by parathyroid hormone (PTH; 21, 22) and calcium (23, 24). In contrast, there is a wide variation in the serum concentration of 25OHD_3, which reflects the availability of vitamin D_3. Our group has recently found an association between prostatic cancer risk and low serum 25OHD_3 concentration (25). Since the expression of 1α-hydroxylase and 24-hydroxylase has been recently documented in extrarenal tissues, including colon (26), prostatic epithelial cells (27), macrophages (28), and keratinocytes (14), the importance of the local metabolism of 25OHD_3 as a precursor is arousing interest. Therefore, the fluctuation of 25OHD_3 serum concentration might be crucial in the regulation of cell proliferation and differentiation of extrarenal tissues.

In studies concerning the action and metabolism of vitamin D_3 compounds in the prostate, the major focus has been the epithelial compartment of this organ. Less attention has been paid to the potential role of the stromal compartment in mediation and modification of biological effects and activities of vitamin D_3 metabolites. However, epithelial and stromal cells are present in approximately equal numbers (29) in human prostate and stromal cells are the first to face hormonal agents derived from circulation. Thus, stromal cells may play a central role in the metabolism and action of vitamin D_3 compounds in prostate organ. However, there is cumulative evidence that the prostatic stromal compartment plays a critical role not only in the regulation of normal epithelial differentiation but also the progression of tumorigenesis (30). Studies in vitro and in vivo have shown that prostatic fibroblasts can affect tumor cell growth and progression, the type and the extent of the response depending on both degree of malignancy of epithelial cells and pathologic state of fibroblast origin. For instance, in the study by Olumi et al. prostatic fibroblasts derived from malignant human tissue were found to enhance growth, retard cell death, and alter histology of initiated but not tumorigenic human epithelial cells (31). Normal prostatic fibroblasts have also been reported to reduce death of LNCaP cells in vitro coculture and in vivo xenografts systems (32). Hence, suppression or stimulation of prostatic fibroblast could affect cancer cell growth.

There are few studies on the action of vitamin D_3 metabolites on the prostatic stromal cells. The issue of 1α,25-$(OH)_2D_3$ action in vitro is controversial. 1α,25-$(OH)_2D_3$ was suggested to exhibit either a stimulatory effect (33) or a less inhibitory effect on human prostatic stromal cells than on

the epithelial cells (4). 1α,25-$(OH)_2D_3$ has been found in vivo to increase the stromal proliferation of the normal rat prostate in the absence of testosterone (34), but prepubertal administration of 1α,25-$(OH)_2D_3$ inhibited the exogenous dihydrotestosterone (DHT) action on stimulating stromal proliferation in the rat prostate (35). In addition, the finding that prostatic stromal cells do not express 1α-hydroxylase mRNA by RT-PCR seems to preclude the study of $25OHD_3$ action on those cells (27). To better understand this issue, we studied the biological activity of $25OHD_3$ and 1α,25-$(OH)_2D_3$ on transcriptional activation of 24-hydroxylase and cell growth in primary stromal cells of the human prostate. Our results raise several important aspects in reference to the potential use of different vitamin D_3 metabolites in the prevention and treatment of prostate cancer.

MATERIALS AND METHODS

Reagents

1α,25-$(OH)_2D_3$ and $25OHD_3$ were obtained from Leo Pharmaceuticals (Ballerup, Denmark). VID400 and SDZ88-357 were kindly provided by Anton Stuetz (Novartis Research Institute, Vienna, Austria). Tissue culture media were purchased from Sigma-Aldrich (Saint Louis, MO). All other reagents, except where indicated, were purchased from Gibco BRL (Life Technologies, Paisley, Scotland).

Cell culture

Human prostate cancer cells LNCaP clone FGC and DU145, obtained from the American Type Culture Collection (ATCC, Manassas, VA) were routinely maintained in 75 cm^2 flasks with phenol red-free RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in humidified atmosphere of 5% CO_2 in air. To deplete endogenous steroids, the medium was changed to one with 10% dextran-treated charcoal-stripped fetal bovine serum (DCC-FBS) 4–5 days before starting the experiments.

Tissues

Two primary cultures, designated P29SN and P32S, were derived from a normal area of prostatic carcinoma and adenoma, respectively. The use of prostate tissue was approved by the local ethical committee, and informed consent was obtained from both subjects.

Isolation and culture of stromal cells

Stromal cell cultures were established essentially according to previously described methods (36). Tissue samples were minced into fragments not larger than 3 mm^3 and subjected to enzymatic dissociation. After overnight digestion at 37°C with 0.05% collagenase A (P32S) or 0.05% collagenase/dispase (P29SN), the partly digested tissue was centrifuged and digestion of the pellet was continued with fresh 0.1% collagenase A at 37°C until isolated glands could be observed. Epithelial acini were separated from the stromal fraction by centrifugation at 50 g. The stromal fraction was carefully rinsed with culture medium and transferred to a 75 cm^2 culture flask. The primary stromal cells and serial cultures were maintained in phenol red-free DMEM/F12 medium, supplemented with 5% DCC-FBS, and 5 μg/ml insulin and antibiotics

(penicillin 100 units/ml, streptomycin 100 µg/ml, amphotericin B 2.5 µg/ml) at 37°C in humidified atmosphere of 5% CO_2 in air. Cells used in the experiments were from passages 6 to 8.

Characterization of the primary cultures

For immunohistochemical analysis cells from each primary culture were grown on 4-well glass slides (Lab-Tek II Chamber Slide, Nalge Nunc, Naperville IL) until subconfluent. The cells were then fixed with 2% formaldehyde for 20 min at room temperature and thereafter permeabilized with pre-chilled (–20°C) 94% ethanol for 10 min on ice.

Mouse monoclonal anti-human antibodies were used to immunohistochemically characterize the stromal primary cultures. Antibodies against vimentin (1:200), desmin (1:100), smooth muscle actin (1:100), and cytokeratins 5/6 (1:100) and 18 (1:50) were purchased from Dako (Glostrup, Denmark). Antibody against cytokeratins 14 (1:200) was from Novocastra (Newcastle, UK), and those against fibronectin (1:50) and cytokeratin 8 (1:50) from Santa Cruz (Santa Cruz, CA). Rat monoclonal anti-VDR antibody (1:200) was from Neo Markers (Fremont, CA). Controls included omission of the primary antibodies and staining with nonimmunized mouse IgG. Normal rat IgG (Santa Cruz Biotechnology) was used as control of VDR staining.

The staining was performed with a broad-spectrum Zymed Histostain-Plus kit (Zymed Laboratories, South San Francisco, CA) with the following modifications to the manufacturer's instructions: primary antibodies were incubated overnight at 4°C and biotinylated second antibody 20 min RT. All washings were repeated three times, 5 min each.

Cell growth assay

Both P29SN and P32S cells at the seventh passage were cultured under the conditions described earlier. Cell growth assays were performed in 96-well culture plates seeding 1000 cells/well in a volume of 200 µl medium. Attachment was allowed for 24 h. Then the cells were treated with 100, 250, and 1000 nM of $25OHD_3$ or 10 nM of $1\alpha,25\text{-}(OH)_2D_3$. Both control cells and treated cells received ethanol vehicle at a concentration of 0.1%. Media were changed, and treatments were renewed every 48 h. Relative cell numbers were quantified at 0, 3, 5, 7, 9, and 11 days by using crystal violet assay (37). Briefly, cells were fixed with 11% glutaraldehyde, washed with de-ionized water, air-dried, stained with 0.1% crystal violet, washed with de-ionized water and air-dried. Then 10% acetic acid was added, and a Victor 1420 Multilabel Counter (Wallac, Turku, Finland) was used for the optical density measurements of extracts at a wavelength of 590 nm. Two separate experiments were performed in which six determinations were used for each treatment.

To verify the results obtained by crystal violet assay, the cell growth was analyzed by counting cell numbers. P29SN cells at the eighth and ninth passages seeded in 24-well plates at a density of 5758 cells per well in 1 ml medium were treated with vehicle (0.1% ethanol), 100 nM, 250 nM of $25OHD_3$ or 10 nM of $1\alpha,25\text{-}(OH)_2D_3$. Media were changed and treatments were renewed every 48 h. At Day 9, cells were trypsinized and pelleted. Cell numbers were counted in a Burker chamber (Assistent, Sondheim, Germany). The experiments were performed three times independently by two people, and the results are expressed as percent of control (mean ± SD).

Immunoblotting

The subconfluent cells were trypsinized and pelleted. Cell lysate protein was prepared by using M-Per™ mammalian protein extraction reagent (Pierce, Rockford, IL) following the manufacturer's instructions. Protein concentrations were measured using BCA protein assay kit (Pierce). Cell lysate was subjected to sodium dodecyl sulfate-PAGE (SDS-PAGE) by using a 7.5% gel. Protein bands were transferred to nitrocellulose transfer membranes (0.45 μm pore; Schleicher and Schuell, Germany). After blocking of nonspecific binding sites with 20% nonfat milk in Tris-HCl buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature for 1 h, the membranes were incubated with anti-mouse 25-hydroxyvitamin D_3-1α-hydroxylase antibody (The Binding Site Ltd. Birmingham, UK; 23) at a 1:500 dilution in TBS-T containing 0.1% nonfat milk at 4°C overnight. After washing with TBS-T, the membranes were incubated with secondary antibody (horseradish peroxidase-conjugated; Zymed) at a 1:4000 dilution in TBS-T containing 0.1% nonfat milk at room temperature for 1 h. The blots were detected by enhanced chemiluminescence reagents (ECL, UK) and exposed to X-ray film for 2 min. The control experiment included pre-saturation of the primary antibody with an excess of the immunizing peptide (mouse amino acid sequence 266 to 289: R-H-V-E-L-R-E-G-E-A-A-M-R-N-Q-G-K-P-E-E-D-M-P-S) (38).

1α-Hydroxylase activity assays

Eighth-passage P29SN cells were seeded in 25 cm^2 flasks in 3 ml of complete growth medium. After 48 h of incubation, the medium was replaced with fresh medium containing 250 nM $25OHD_3$ and/or 1000 nM SDZ88-357. At 4 h, the media and cells were collected for quantitation of $1\alpha,25\text{-}(OH)_2D_3$. All samples were pre-purified by using the acetonitrile-C18 Sep-Pak Cartridge (Waters, Ireland; 39), followed by separation of the metabolites by high performance liquid chromatography (Pharmacia LKB HPLC pump 2248, VWM 2141, Uppsala, Sweden). The concentrations of $1\alpha,25(OH)_2D_3$ were quantified by radioreceptor assay (40). The corresponding protein concentrations were measured by using BCA protein assay kit (Pierce). Enzymatic activity was expressed as fmol/mg protein/h. Data are expressed as means (± SD) of five repeats.

RNA isolation

The subconfluent cells were treated with vehicle (ethanol, final concentration 0.05%) or the compounds were noted at the concentrations indicated and for 6, 24, and 48 h. The ethanol concentration was equal in controls and hormone-treated samples.

Total cellular RNAs were isolated using Trizol reagent (Gibco BRL, Life Technologies, Grand Island, NY) following the manufacturer's instructions. Total RNA amounts were quantified by measuring absorbance at 260 nm. The OD_{260}/OD_{280} nm absorption ratio was always greater than 1.93. Denaturing agarose gel electrophoresis was performed to verify the integrity of RNA. The intensity of the 28S rRNA band was more than twice that of the 18S rRNA band stained by ethidium bromide.

Primer Design

As recommended in the manufacturer's protocol, primers were designed by using Primer Express v2.0 software (Perkin-Elmer Applied Biosystems, Foster City, CA) to ensure suitability for the ABI Prism 7000 sequence detection system and the reaction parameters. To confirm the specificity of the primer sequences, we performed BLASTN searches. All primers were designed to be intron-spanning to preclude amplification of genomic DNA. To normalize the amount of sample cDNA added to the reaction, human acidic ribosomal phosphoprotein P0 (RPLP0) was used as the endogenous control. RPLP0 is ubiquitously expressed and is considered to be a reliable endogenous control. For RPLP0 (NM_001002) amplification, the forward primer was 5′-AATCTCCAGGGGCACCATT-3′, which corresponds to base 515–533; the reverse primer was 5′-CGCTGGCTCCCACTTTGT-3′, which corresponds to base 588–571. For CYP24 (NM_000782) amplification, the forward primer was 5′-GCCCAGCCGGGAACTC-3′, which corresponds to base 1907–1922; the reverse primer was 5′-AAATACCACCATCTGAGGCGTATT-3′, which corresponds to base 1968–1945. For CYP27B1 (NM_000785) amplification, the forward primer was 5′-TTGGCAAGCGCAGCTGTAT-3′, which corresponds to base 1409–1427; the reverse primer was 5′-TGTGTTAGGATCTGGGCCAAA-3′, which corresponds to base 1484–1464. All sequence-specific oligonucleotide primers were synthesized by TAG Copenhagen A/S (Copenhagen, Denmark).

cDNA synthesis and quantitative real-time polymerase chain reaction (PCR)

The total RNA from each sample was reverse-transcribed using a high-capacity cDNA achieve kit (Perkin-Elmer Applied Biosystems) following the manufacturer's instructions. The experimental protocol was as follows: 10 min at 25°C followed by 120 min reverse transcription at 37°C. All PCR reactions were performed in MicroAmp optical 96-well reaction plates using an SYBR Green Master Mix kit (Perkin-Elmer Applied Biosystems) on an ABI Prism 7000 sequence detection system (Perkin-Elmer Applied Biosystems). The thermal cycling conditions consisted of a 10 min polymerase activation/initial denaturation at 95°C and 45 cycles with a 95°C denaturation for 15 s and a 60°C annealing/extension for 1 min. Detection of accumulated fluorescent products was performed at the end of the extension step of each cycle. To verify the specific products, dissociation curve analysis was performed after 45 cycles.

Serial dilutions of cDNA from the cells treated with 10 nM 1α,25-$(OH)_2D_3$ for 24 h were made to generate the standard curves of endogenous control and target genes. The calibrator sample used in the data analysis was the untreated sample. The data were quantified by the standard curve method with ABI Prism SDS Data Analysis software. The relative expression level of the target gene was calculated by using amplification efficiencies obtained from the standard curves and Ct values as described previously (41).

PCR was performed as duplicates for each sample. The means of the results were expressed as relative expression levels compared with the calibrator. This procedure was repeated for 2–4 independent samples and the result is given as the mean ± SD.

RESULTS

Characterization of the primary cultures

Both primary cultures showed similar staining characteristics. An extensive staining for vimentin (Fig. 1*A*) and fibronectin (Fig. 1*B*) was seen with over 99% of cells positive for these markers. Less than 5% of the cells present expressed smooth muscle actin (Fig. 1*C*) and less than 2% expressed desmin (Fig. 1*D*). There was no specific staining with anti-cytokeratins 8 and 18. Stainings with anti-cytokeratins 5/6 and 14, as well as PBS, were negative (data not shown). A positive immunostaining for VDR (Fig. 1*E*) was detected in the discrete foci of cell nuclei. The control staining for VDR was negative. The data indicate that the vast majority of both primary prostatic cultures are fibroblasts in phenotype.

The effects of 25OHD$_3$ and 1α,25-(OH)$_2$D$_3$ on the growth of primary prostatic stromal cells

To study the action of vitamin D$_3$ on the proliferation of both P29SN and P32S primary cultures, two independent crystal violet assays were performed. The growth of P29SN cells was significantly inhibited when treated with 250 and 1000 nM of 25OHD$_3$ and 10 nM of 1α,25-(OH)$_2$D$_3$ (Fig. 2*A*). The antiproliferative action of 25OHD$_3$ was dose-dependent. Compared with the controls, the relative cell growth at Day 9 treated with 100 nM, 250 nM, 1000 nM of 25OHD$_3$, and 10 nM of 1α,25-(OH)$_2$D$_3$ was 98±25% (P>0.05), 70 ± 8% (P<0.01), 51 ± 6% (P<0.0001) and 62 ± 9% (P<0.001), respectively (Fig. 2*C*).

The growth of P32S cells was not inhibited by 10 nM of 1α,25-(OH)$_2$D$_3$ but significantly inhibited by 100 nM (P<0.01), 250 nM (P<0.01), and 1000 nM (P<0.0001) of 25OHD$_3$ (Fig. 2*B*). Compared with the controls, the relative cell growth at Day 9 treated with 100, 250, and 1000 nM of 25OHD$_3$ and 10 nM of 1α,25-(OH)$_2$D$_3$ was 81 ± 12, 77 ± 9, 60 ± 7, and 95 ± 20%, respectively. Collectively, the results demonstrate that P29SN cells exhibited growth suppression only with pharmacological concentration of 1α,25-(OH)$_2$D$_3$ and that P32S cells were unresponsive to this metabolite. However, both primary cells responded effectively to physiological concentrations of 25OHD$_3$ with respect to the inhibition of cell growth.

To verify the results above, cell number counting method was applied. Compared with the controls, the relative growth of P29SN cells at Day 9 treated with 100 and 250 nM of 25OHD$_3$ and 10 nM of 1α,25-(OH)$_2$D$_3$ was 139 ± 16% (P=0.050), 78 ± 5% (P=0.018), and 68 ± 8% (P=0.023), respectively.

Expression of 1α-hydroxylase protein

Immunoblotting analysis using an anti-mouse 25-hydroxyvitamin D$_3$-1α-hydroxylase antibody showed a clear single band at 56 KD in both primary cultures, which is the size of 1α-hydroxylase protein (Fig. 3, Lanes 5 and 7). In DU145 cells, a weak band of 56 KD was found (Fig. 3, Lane 3). No signal was seen in the pre-saturation controls (Fig. 3, Lanes 4, 6, and 8). However, in LNCaP cells, a very weak band of 56 KD was found along with a nonspecific band at 64.6 KD (Fig. 3, Lane 1), which did not disappear in the presaturation control (Fig. 3, Lane 2).

1α-Hydroxylase activity in primary prostatic stromal cells

To determine whether the primary prostatic stromal cells can produce 1α,25-$(OH)_2D_3$, we performed 1α-hydroxylase activity assay. 1α-Hydroxylase activity in P29SN cells was 30 ± 29 fmol/mg protein/h (n=5). However, the concentration of 1α,25-$(OH)_2D_3$ in culture medium and cells was much lower than physiological concentration (50 PM). When the cells received 250 nM $25OHD_3$ in the presence of 1000 nM SDZ88-357, a specific 1α-hydroxylase inhibitor (42), 1α-hydroxylase activity was 7 ± 36 fmol/mg protein/h (n=5), which indicates that SDZ88-357 can effectively inhibit 1α-hydroxylase activity.

The presence of 1α-hydroxylase mRNA and its regulation by $25OHD_3$ and 1α,25-$(OH)_2D_3$ in primary prostatic stromal cells

In both P29SN and P32S cells, quantitative real-time RT-PCR showed a detectable and similar level of 1α-hydroxylase mRNA. Among these, only in P29SN cells did the use of $25OHD_3$ in concentration of 100 nM at 6 h cause statistically significant up-regulation of 1α-hydroxylase mRNA (2 ± 0.3-fold, P<0.05, Fig. 4).

Induction of 24-hydroxylase mRNA by $25OHD_3$ and 1α,25-$(OH)_2D_3$ is dose-dependent in primary prostatic stromal cells

To explore the transcriptional regulation by $25OHD_3$ and 1α,25-$(OH)_2D_3$ in stromal cells, we measured 24-hydroxylase mRNA using quantitative real-time RT-PCR. 1α,25-$(OH)_2D_3$ at a physiological concentration (0.1 nM) had no effect on the expression of 24-hydroxylase mRNA at 6 h in either primary culture. 1α,25-$(OH)_2D_3$ (10 nM) dramatically increased the mRNA level of 24-hydroxylase in P29SN and P32S cells. (Fig. 6*A*).

Similarly, $25OHD_3$ exhibited a dose-dependent induction of 24-hydroxylase mRNA. At 100 nM, $25OHD_3$ increased the mRNA level of 24-hydroxylase 2.27 ± 0.32-fold (P>0.05) in P29SN cells and had no effect in P32S cells, whereas 250 and 1000 nM $25OHD_3$ enhanced the mRNA level of 24-hydroxylase in P29SN (200 ± 5-fold, P<0.01 and 12000 ± 220-fold, P<0.01, respectively; Fig. 5*A*) and P32S cells (4±0.5-fold, P<0.05 and 660 ± 5-fold, P<0.0001, respectively, Fig. 5*B*). These data indicate that 1α,25-$(OH)_2D_3$ at pharmacological concentration and $25OHD_3$ at physiological concentration can induce 24-hydroxylase mRNA expression.

The time-course of 24-hydroxylase mRNA expression after $25OHD_3$ and 1α,25-$(OH)_2D_3$ in primary prostatic stromal cells

The cells were treated with 10 nM 1α,25-$(OH)_2D_3$ and 250 nM $25OHD_3$ for 6, 24, and 48 h, and 24-hydroxylase mRNA was quantified by using RT-PCR. Both 10 nM 1α,25-$(OH)_2D_3$ and 250 nM $25OHD_3$ time-dependently induced the expression of 24-hydroxylase mRNA. In P29SN cells, 10 nM 1α,25-$(OH)_2D_3$ increased 24-hydroxylase mRNA level 6900 ± 500-fold (P<0.001), 14600 ± 800-fold (P<0.0001), and 2900 ± 500-fold (P<0.01) at 6, 24, and 48 h, respectively (Fig. 6*A*). Similarly, in P32S cells, 10 nM 1α,25-$(OH)_2D_3$ increased 24-hydroxylase mRNA level 4200 ± 1600-fold (P>0.05), 34000 ± 200-fold (P<0.01), and 18000 ± 200-fold (P<0.01) at 6, 24, and 48 h, respectively (Fig. 6*A*). In P29SN and P32S cells, treatment with 250 nM $25OHD_3$

caused a 200 ± 5-fold (P<0.01) and 4 ± 0.5-fold (P<0.05) stimulation at 6 h, a 140 ± 10-fold (P<0.05) and 7 ± 2-fold (P>0.05) stimulation at 24 h, a 90 ± 0.2-fold (P<0.01) and 5 ± 0.7-fold (P<0.05) stimulation at 48 h, respectively (Fig. 6*B*). Thus, the induction of 24-hydroxylase by 1α,25-$(OH)_2D_3$ appeared to be much stronger in P32S cells as compared with P29SN cells, whereas 25OHD_3 caused much weaker induction of 24-hydroxylase in P32S cells than in P29SN cells.

The effect of the inhibition of 24-hydroxylase enzyme activity on gene expression by 25OHD_3 and 1α,25-$(OH)_2D_3$ in primary prostatic stromal cells

An inhibitor of 24-hydroxylase, VID400, was used at a concentration of 100 nM (42). In P29SN cells, VID400 increased the induction of 24-hydroxylase mRNA by 10 nM 1α,25-$(OH)_2D_3$ from 7000 ± 96-fold (without VID400) to 13000 ± 1000-fold, which was a significant difference (P<0.05, Fig. 7). Similarly, in P32S cells, treatment with 10 nM 1α,25-$(OH)_2D_3$ alone caused a 4200 ± 1600-fold stimulation, which was enhanced by 2.5-fold (P>0.05) in the presence of VID400 (Fig. 7). Furthermore, 250 nM 25OHD_3 with VID400 exhibited an 8.3-fold (P<0.05) and 60-fold (P<0.05) stimulatory effect compared with 250 nM 25OHD_3 alone in P29SN (Fig. 8*A*) and P32S cells (Fig. 8*B*), respectively. These data indicate that by inhibiting 24-hydroxylase activity, more 1α,25-$(OH)_2D_3$ and 25OHD_3 are available in the induction of 24-hydroxylase mRNA.

The effect of the inhibition of 1α-hydroxylase and 24-hydroxylase on gene expression by 25OHD_3 in primary prostatic stromal cells

To investigate whether 25OHD_3 is active without 1α-hydroxylation, we added a specific inhibitor for 1α-hydroxylase, SDZ88-357, which has been shown earlier to inhibit 1α-hydroxylase activity. When cells were treated with 250 nM 25OHD_3 and 1000 nM SDZ88-357 in combination, 24-hydroxylase mRNA was induced 820 ± 180-fold in P29SN cells (Fig. 8*A*) and 8±1.5-fold in P32S cells (Fig. 8*B*), indicating that 250 nM 25OHD_3 is four times (P>0.05) and two times (P>0.05) more effective than in the absence of 1α-hydroxylase inhibitor in P29SN and P32S cells, respectively. When the cells were exposed to 250 nM 25OHD_3 with 100 nM VID-400 and 1000 nM SDZ88-357 in combination, 24-hydroxylase mRNA was induced 1200±220-fold in P29SN cells (Fig. 8*A*) and 16±4-fold in P32S cells (Fig. 8*B*), which were more pronounced inductions than in the absence of inhibitors (200 ± 5-fold in P29SN cells and 4 ± 0.5-fold in P32S cells). These results suggest that 250 nM 25OHD_3 can induce 24-hydroxylase mRNA and 1α-hydroxylation is not a prerequisite for its hormonal activity of 25OHD_3.

Induction of 24-hydroxylase mRNA by 25OHD_3 and 1α,25-$(OH)_2D_3$ in LNCaP cells

To determine whether this phenomenon occurs in LNCaP cells, a prostatic epithelial cell line expressing an extremely low level of 1α-hydroxylase protein shown in Fig. 3, we studied the effect of 25OHD_3 and 1α,25-$(OH)_2D_3$ on 24-hydroxylase mRNA. LNCaP cells were treated with 10 nM 1α,25-$(OH)_2D_3$ and 1000 nM 25OHD_3 for 6, 24, and 48 h, and 24-hydroxylase mRNA was quantified by using RT-PCR. Both 10 nM 1α,25-$(OH)_2D_3$ and 1000 nM 25OHD_3 time-dependently induced the expression of 24-hydroxylase mRNA. 1α,25-$(OH)_2D_3$ (10 nM) increased 24-hydroxylase mRNA level 1218 ± 220-fold (P>0.05), 4294 ± 16-fold (P<0.01), and

2513 ± 118-fold (P<0.05) at 6, 24, and 48 h, respectively (Fig. 9*A*). 25OHD$_3$ (1000 nM) increased 24-hydroxylase mRNA level 3730 ± 342-fold (P<0.05), 11618 ± 199-fold (P<0.01), and 9300 ± 906-fold (P<0.05) at 6, 24, and 48 h, respectively (Fig. 9*A*). However, neither 1α,25-(OH)$_2$D$_3$ at a physiological concentration (0.1 nM) nor 100–250 nM 25OHD$_3$ affected the expression of 24-hydroxylase mRNA at 24 h in LNCaP cells (Fig. 9*B*). These results demonstrate that 1000 nM 25OHD$_3$ specifically induced 24-hydroxylase mRNA expression in LNCaP cells, which are less sensitive to 25OHD$_3$ than primary prostatic stromal cells. It is important to note that 1000 nM 25OHD$_3$ was found to be more potent in increasing 24-hydroxylase mRNA expression than 10 nM 1α,25-(OH)$_2$D$_3$.

DISCUSSION

Although prostatic epithelial cells have been studied as targets for the antiproliferative action of 1α,25-(OH)$_2$D$_3$, the differentiation of prostatic epithelial cells is determined by the underlying stromal cells, which may mediate the effect of hormones on the epithelial cells (30, 43). Our primary goal was to investigate the biological activities of 25OHD$_3$ and 1α,25-(OH)$_2$D$_3$ and their metabolism in prostatic stromal cells. Therefore, we established two primary human prostatic stromal cell cultures, isolated from a normal area of prostatic carcinoma (P29SN) and adenoma (P32S). The primary cultures were almost exclusively fibroblasts in type since the fibroblast markers were found in more than 99% of the cells. The cells were vitamin D$_3$-responsive, since they expressed VDR. Therefore, they were a suitable model to study vitamin D$_3$ action.

Using this model, we demonstrate a direct action of both 1α,25-(OH)$_2$D$_3$ and 25OHD$_3$ on human prostatic stromal cells. The biological activity of 100–250 nM 25OHD$_3$ (physiological concentrations 20-105 nM) on the inhibition of cell growth and on the stimulation of the 24-hydroxylase mRNA expression is higher than that of 10 nM 1α,25-(OH)$_2$D$_3$, whereas 0.1 nM of 1α,25-(OH)$_2$D$_3$ within a physiological concentration range (48–156 PM) is inactive. When 1α-hydroxylase enzyme activity is suppressed by a specific inhibitor, 250 nM 25OHD$_3$ appears to be more potent (2–4 times) in inducing the expression of 24-hydroxylase mRNA than in the absence of the inhibitor. The reason for the enhanced induction of 24-hydroxylase is not known, but it may be due to missing VDR binding of 1α,25-(OH)$_2$D$_3$ in the presence of 1α-hydroxylase inhibitor. This suggests that 25OHD$_3$ possesses an inherent hormonal activity and that its activation through 1α-hydroxylation is not essential for its biological activity. Interestingly, 10 nM of 1α,25-(OH)$_2$D$_3$ failed to inhibit the growth of P32S cells, whereas 250 nM 25OHD$_3$ reduced their growth, suggesting that the cells are more sensitive to 25OHD$_3$ than to 1α,25-(OH)$_2$D$_3$. The reason for the unresponsiveness of P32S to 1α,25-(OH)$_2$D$_3$ with respect to the growth inhibition may be related to a fast inactivation of 1α,25-(OH)$_2$D$_3$ by 24-hydroxylase. With these cells induction of 24-hydroxylase transcription by 1α,25-(OH)$_2$D$_3$ was found to be much stronger compared with P29SN cells (Fig. 6). It is not known whether the mechanism of the growth-inhibitory effect of 25OHD$_3$ differs from that of 1α,25-(OH)$_2$D$_3$, but both metabolites are known to bind the same nuclear VDR. In vitro studies have demonstrated that 1α,25-(OH)$_2$D$_3$ has 667 times more binding affinity for the chick intestinal VDR than 25OHD$_3$ (44), 63 times more in LNCaP cells (45). In our present study, the biological activities of 25OHD$_3$ and 1α,25-(OH)$_2$D$_3$ showed a 25- to 100-fold concentration difference in inhibiting cell proliferation and inducing 24-hydroxylase gene expression. In view of the fact that the serum concentrations

of $25OHD_3$ are ~1000-fold greater than those of $1\alpha,25\text{-}(OH)_2D_3$, the biological activity of the circulating $25OHD_3$ is significant.

To exclude the inactivation of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ and the minor biological activity of natural metabolites with 24-hydroxyl group (46), we applied 24-hydroxylase inhibitor. The inhibitor increased the activity of the hormones suggesting that there was a significant inactivation of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ in our primary cultures.

To our knowledge, we have demonstrated for the first time the expression of 1α-hydroxylase detected by quantitative real-time RT-PCR and immunoblotting in prostatic stromal cells. In an early report (27), 1α-hydroxylase mRNA was absent from prostatic stromal cells by RT-PCR perhaps because of the low sensitivity of the traditional RT-PCR. Interestingly, our results indicate that the stromal cells express more 1α-hydroxylase protein than DU145 cells. However, the activity of this enzyme in stromal cells was low, ~40- to 100-fold less compared with that reported in normal epithelial cells (47). LNCaP cells express a very low level of 1α-hydroxylase due to a defect in promoter activity (48). Our finding that $25OHD_3$ can also induce 24-hydroxylase expression in these cells provides further evidence for inherent activity of this vitamin D_3 metabolite. Since the discovery of the process of 1α-hydroxylation in the target organs, $25OHD_3$ has been regarded as a precursor of a local synthesis of $1\alpha,25\text{-}(OH)_2D_3$, which in turn acts as an autocrine/parocrine regulator, for example, in the prostate. In the cell model used, the presence of 1α-hydroxylase allows the production of $1\alpha,25\text{-}(OH)_2D_3$, and it is, therefore, necessary to use 1α-hydroxylase inhibitor when studying $25OHD_3$ action.

On the basis of our results, we propose two distinct vitamin D_3 endocrine systems (Fig. 10). The classical system is involved in calcium and phosphorus regulation based on $1\alpha,25\text{-}(OH)_2D_3$. The synthesis of $1\alpha,25\text{-}(OH)_2D_3$ in kidney is tightly controlled by the hormone itself, PTH, and calcium, through the regulation of 1α-hydroxylase and 24-hydroxylase. As a result, serum concentration of $1\alpha,25\text{-}(OH)_2D_3$ varies within an extremely narrow range. In contrast, calcium metabolism is not sensitive to the physiological serum concentration of $25OHD_3$, which fluctuates within a wide range, depending on the season, whereas $1\alpha,25\text{-}(OH)_2D_3$ serum concentration is not affected by the season (49). The second system is a novel vitamin D_3 endocrine system based on the liver hormone, $25OHD_3$, which regulates cell proliferation and gene expression at a physiological concentration in prostatic stromal cells. It seems that antiproliferative and differentiation actions require $1\alpha,25\text{-}(OH)_2D_3$ at a higher concentration than the serum level, as demonstrated by our study. Hypercalcemic concentrations of $1\alpha,25\text{-}(OH)_2D_3$ are needed to achieve an antiproliferative effect (10, 11). This may also explain epidemiological studies showing that a low serum concentration of $25OHD_3$ is associated with an increased prostate cancer risk (25). On the other hand, the issue concerning physiological concentrations of $25OHD_3$ is still controversial because studies performed on subpopulations living or working in sun-rich environments have shown that exposure to sunlight without dietary supplementation can raise serum $25OHD_3$ values above 200 nM (50). Furthermore, it is likely that 1α-hydroxylase may not be so important, as has been expected in prostate cancer development because sequence variants in the 1α-hydroxylase gene do not correlate with prostate cancer risk (51).

In conclusion, we used the transcriptional induction of 24-hydroxylase and growth-inhibition as indicators of the biological activity of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ in human prostatic stromal

cells. Our data demonstrate several important aspects regarding the role of vitamin D_3 metabolites in the prevention and treatment of prostate cancer. It appeared that $1\alpha,25\text{-}(OH)_2D_3$ is inactive in stromal cells at its physiological concentrations but that pharmacological concentrations are needed for induction of target gene expression. However, $25OHD_3$, which has much lower affinity for the inactivating enzyme, 24-hydroxylase, induces target gene expression and suppresses cell growth in high but physiological concentrations and also induces the expression of the activating enzyme, 1α-hydroxylase. The finding that $25OHD_3$ at a physiological concentration possesses an inherent hormonal activity provides a novel view of the vitamin D_3 endocrine system and suggests a potent anticancer therapy. Further study of the transcriptional regulation of other vitamin D_3 responsive genes by $25OHD_3$ will provide further understanding on the gene specificity of $25OHD_3$.

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Fig. 1

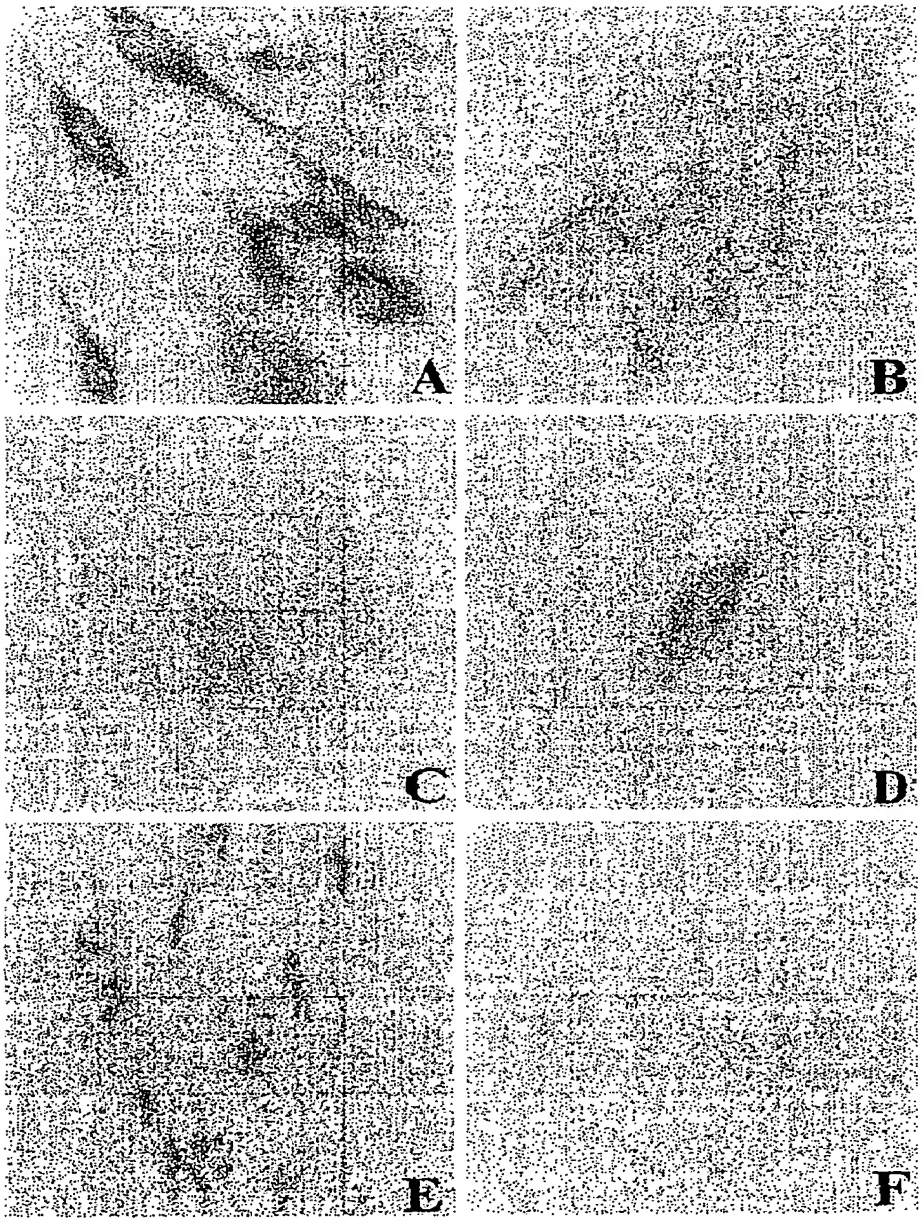

Figure 1. Immunostaining of P29SN cells for vimentin (*A*), fibronectin (*B*), smooth muscle actin (*C*), desmin (*D*), VDR (*E*), and normal mouse IgG (*F*). Immunohistochemical staining was made as described in Materials and Methods. Vimentin and fibronectin are markers for cells with fibroblastic phenotype, whereas smooth muscle actin and desmin are expressed by smooth muscle cells. VDR staining was localized in the discrete foci of cell nuclei.

Fig. 2

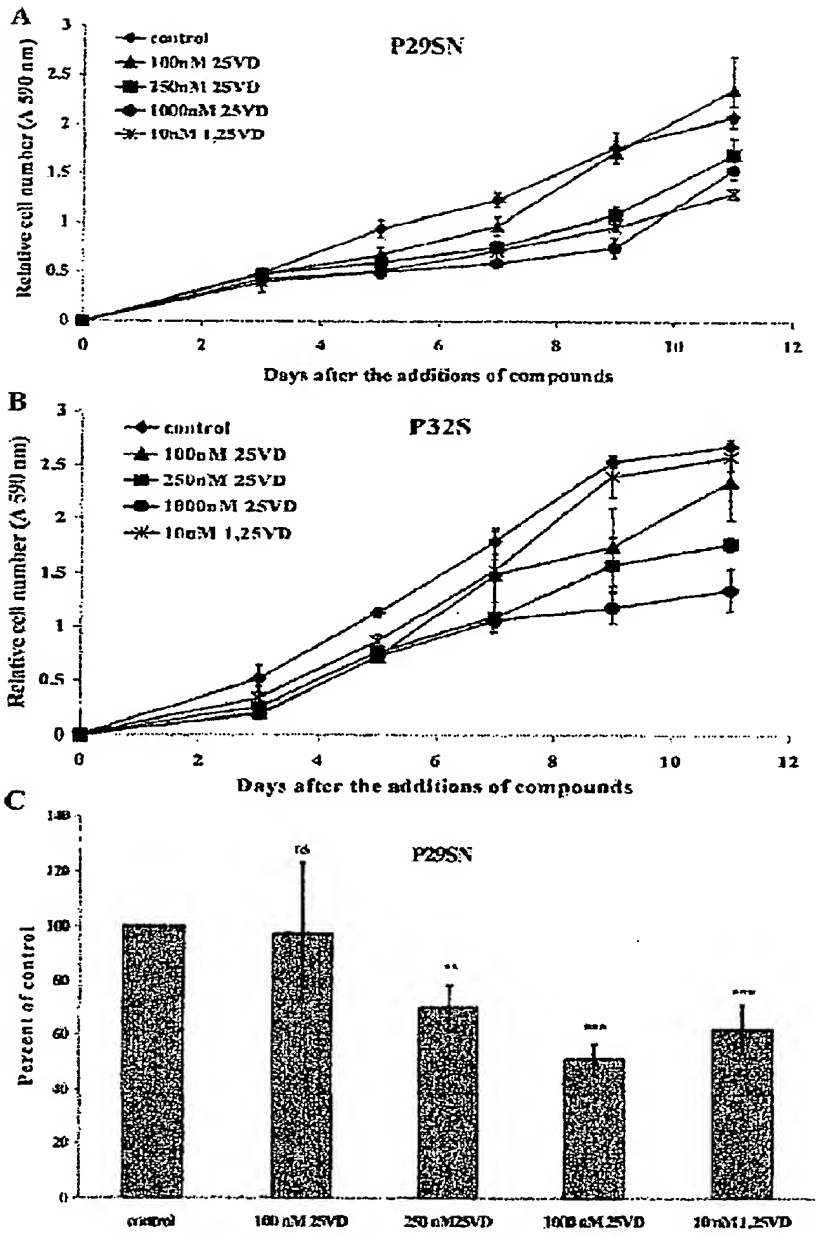

Figure 2. Effects of $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ on the growth of human primary prostatic stromal cells. P29SN (*A*) and P32S (*B*) cells were treated with vehicle (0.1% ethanol), 100, 250, 1000 nM of $25OHD_3$ (25VD) and 10 nM of $1\alpha,25\text{-}(OH)_2D_3$ (1,25VD) for 11 days, and relative cell numbers were determined at Days 0, 3, 5, 7, 9, and 11 using a crystal violet assay. The relative growth of P29SN cells at Day 9 is shown in (*C*). Two separate experiments were performed in which six determinations for each treatment were made. Statistical significance was evaluated by Student's t-test (** $p<0.01$, *** $p<0.001$, ns=not significant, $P>0.05$).

Fig. 3

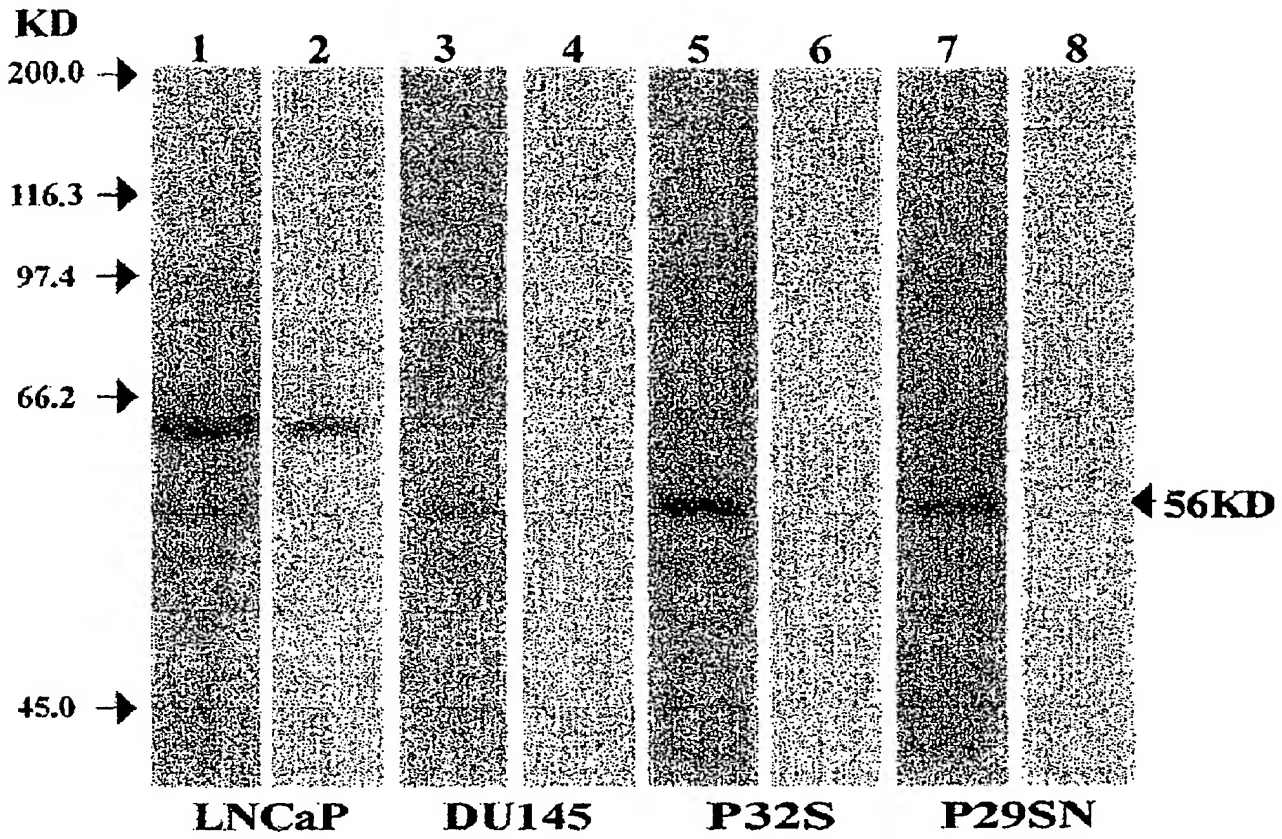

Figure 3. Immunoblotting of 1α-hydroxylase protein. LNCaP (Lane 1, 2), DU145 (Lane 3, 4), P32S (Lane 5, 6), and P29SN (Lane 7, 8) cells were cultured under standard conditions. Cell lysate protein (5 μg) was separated by using 7.5% PAGE and then transferred to nitrocellulose membranes. The blots were detected by ECL and exposed to X-ray film for 2 min. Lanes 2, 4, 6, and 8 are presaturation controls in which the primary antibody was presaturated with an excess of the immunizing peptide. The figure presents data representative of two independent experiments.

Fig. 4

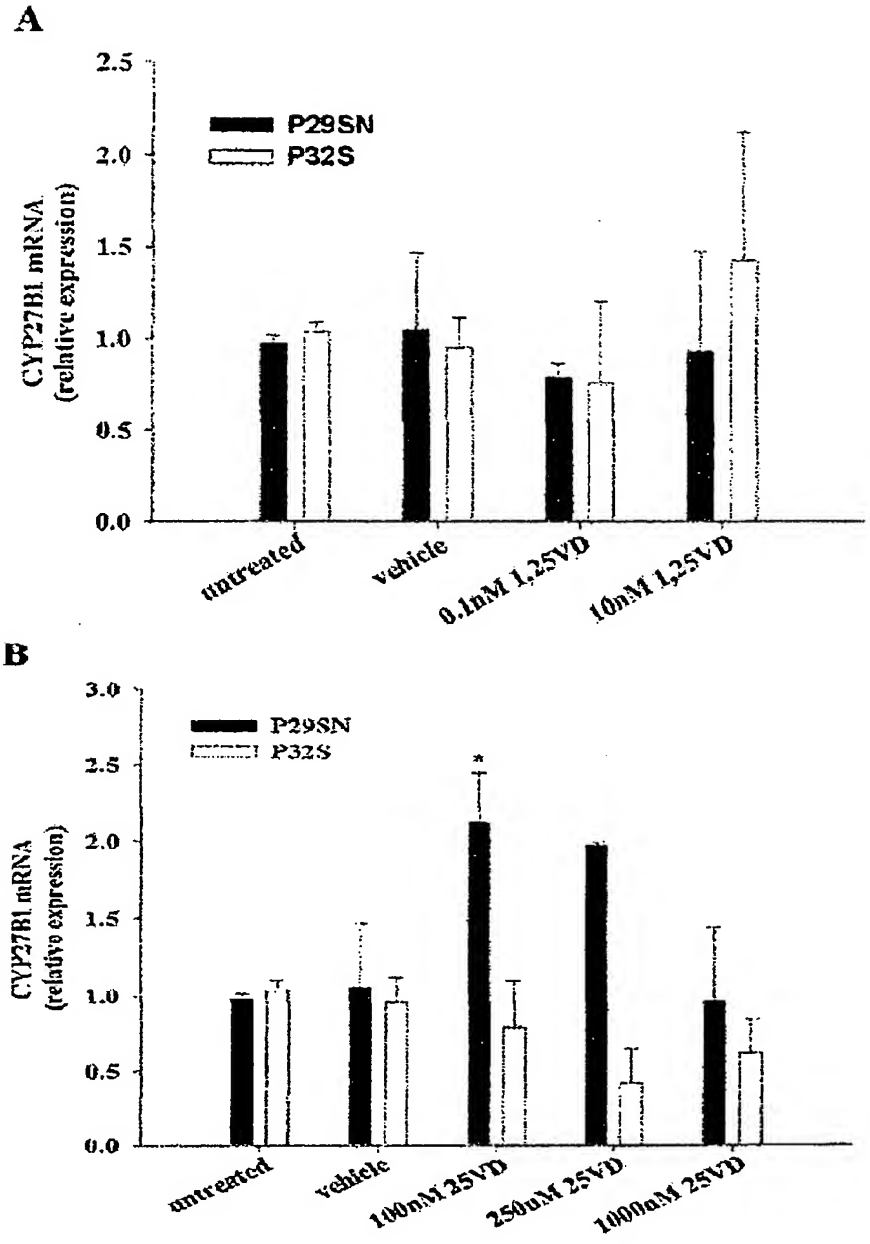

Figure 4. Regulation of 1α-hydroxylase mRNA by 1α,25-$(OH)_2D_3$ and 25OHD_3 in human primary prostatic stromal cells P29SN and P32S. P29SN and P32S cells grown to 70% confluence under standard conditions were treated with vehicle (0.05% ethanol), 1α,25-$(OH)_2D_3$ (1,25VD; *A*) or 25OHD_3 (25VD; *B*) at the concentrations indicated for 6 h. The total cellular RNA was isolated, and 1α-hydroxylase (CYP27B1) mRNA was measured by quantitative real-time RT-PCR. Results are expressed as means (± SD) of two independent experiments performed in duplicate. Statistical significance was evaluated by Student's *t*-test (*P<0.05 vs. vehicle).

Fig. 5

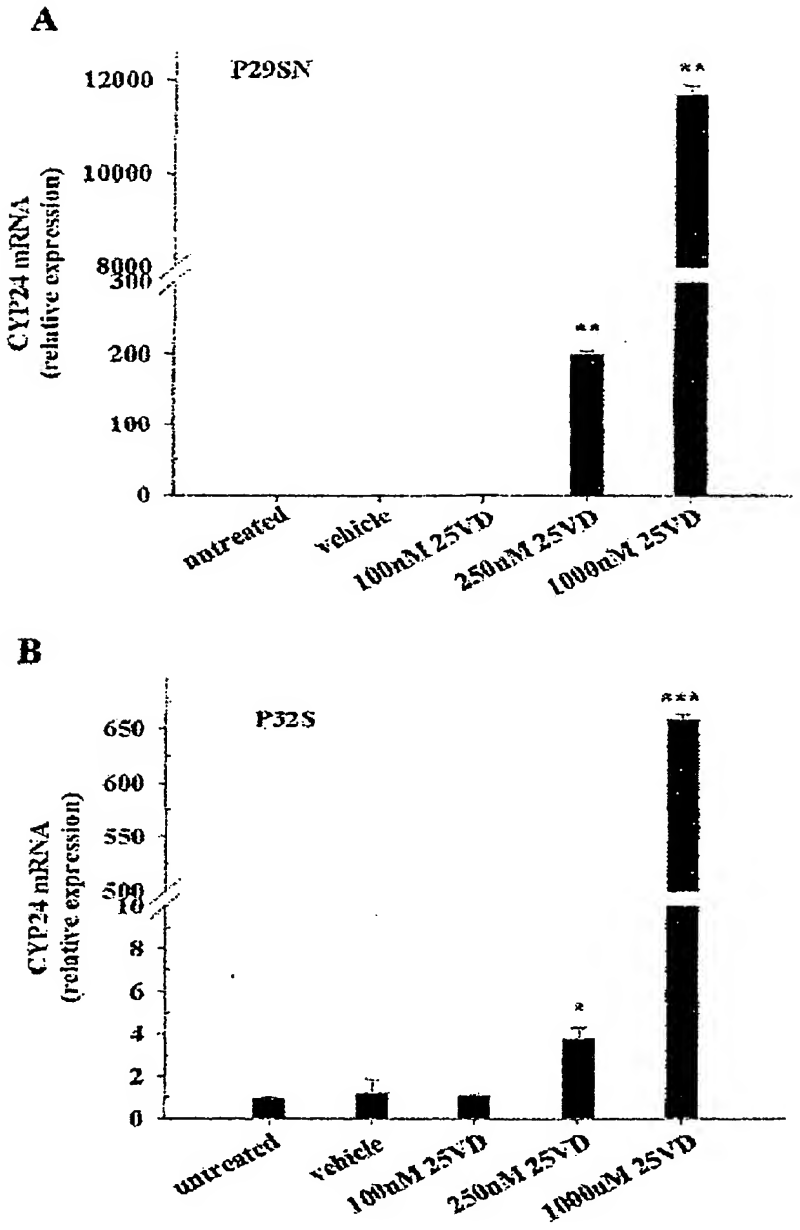

Figure 5. Regulation of 24-hydroxylase mRNA by 25OHD$_3$ in P29SN and P32S cells. P29SN (*A*) and P32S (*B*) cells grown to 70% confluence under standard conditions were treated with vehicle (0.05% ethanol), 25OHD$_3$ (25VD) at the concentrations indicated for 6 h. The total cellular RNA was isolated and 24-hydroxylase (CYP24) mRNA was quantified by quantitative real-time RT-PCR. Results are expressed as means (± SD) of two independent experiments performed in duplicate. Statistical significance was evaluated by Student's *t*-test (*P<0.05, **P<0.01, ***P<0.001 vs. vehicle).

Fig. 6

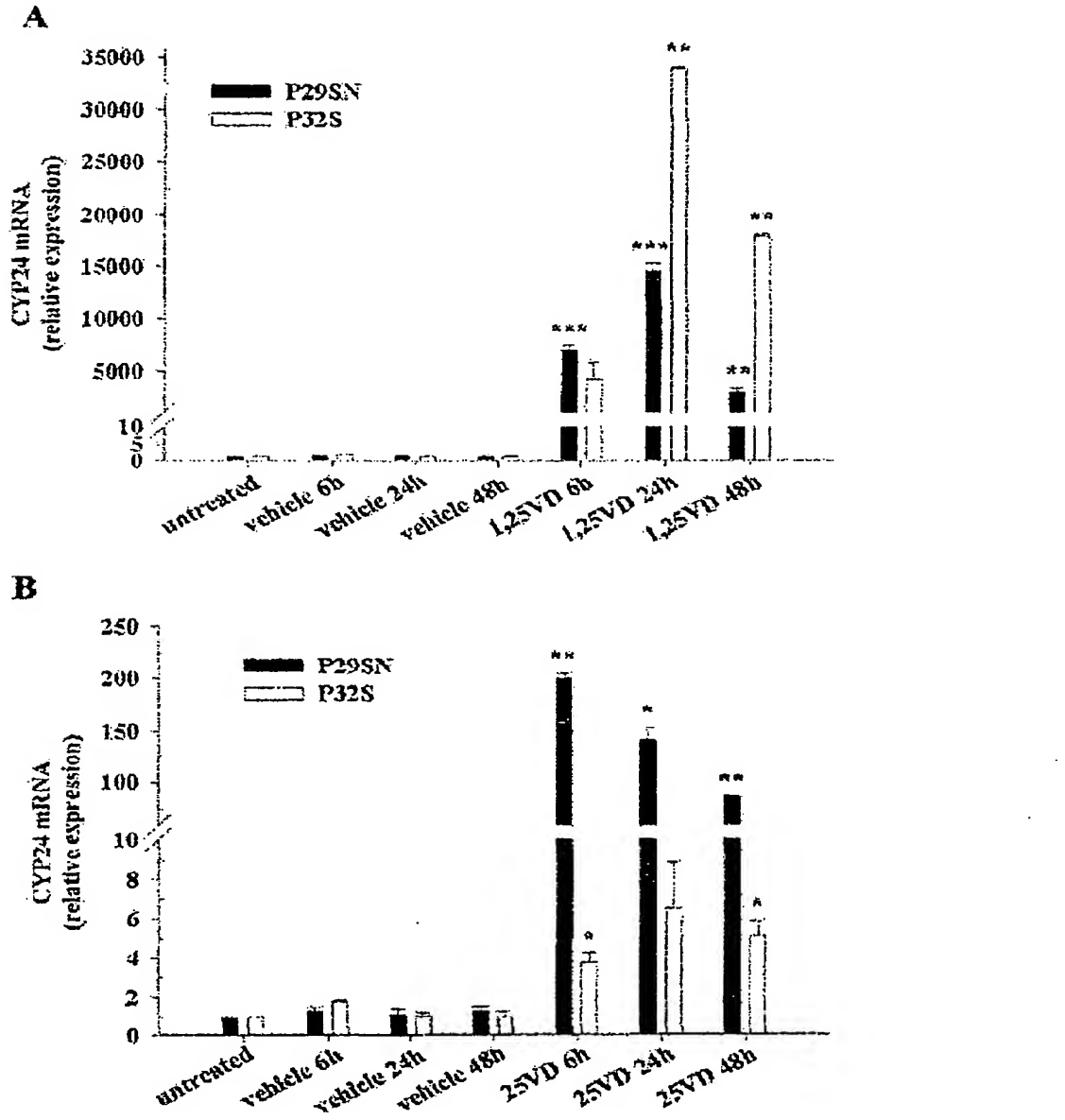

Figure 6. Time-course of 24-hydroxylase mRNA expression in response to 1α,25-$(OH)_2D_3$ and 25OHD_3 in P29SN and P32S cells. P29SN and P32S cells grown to 70% confluence under standard conditions were incubated with vehicle (0.05% ethanol), 10 nM 1α,25-$(OH)_2D_3$ (1,25VD; *A*) or 250 nM 25OHD_3 (25VD; *B*) for 6, 24, and 48 h. 24-hydroxylase (CYP24) mRNA expression was measured by quantitative real-time RT-PCR. Values shown in (*A*) represent the mean ± SD of four independent experiments performed in duplicate. Values shown in (*B*) are the mean ± SD of two independent experiments performed in duplicate.

Fig. 7

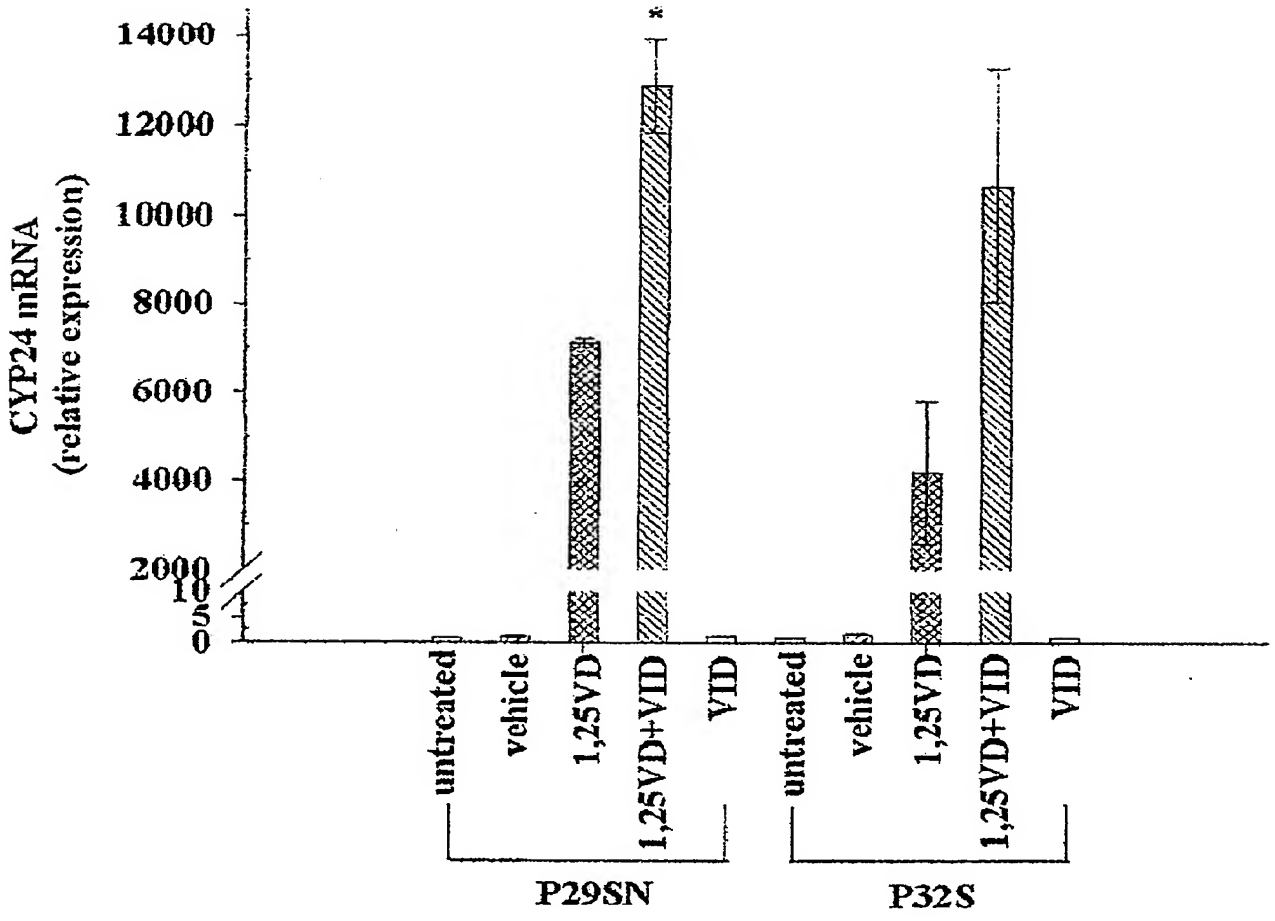

Figure 7. Enhancement of $1\alpha,25\text{-}(OH)_2D_3$ action on the induction of 24-hydroxylase mRNA by inhibiting 24-hydroxylase activity in P29SN and P32S cells. P29SN and P32S cells grown to 70% confluence under standard conditions were incubated with vehicle (0.05% ethanol), 10 nM $1\alpha,25\text{-}(OH)_2D_3$ (1,25VD), or 100 nM VID400 (VID) individually or in combination for 6 h. 24-Hydroxylase (CYP24) mRNA was measured by quantitative real-time RT-PCR. Values from P29SN cells are the mean ± SD of three independent experiments performed in duplicate and those from P32S cells are the mean ± SD of two independent experiments performed in duplicate. VID significantly increased mRNA level compared with 1,25VD alone in P29SN cells (*$P<0.05$).

Fig. 8

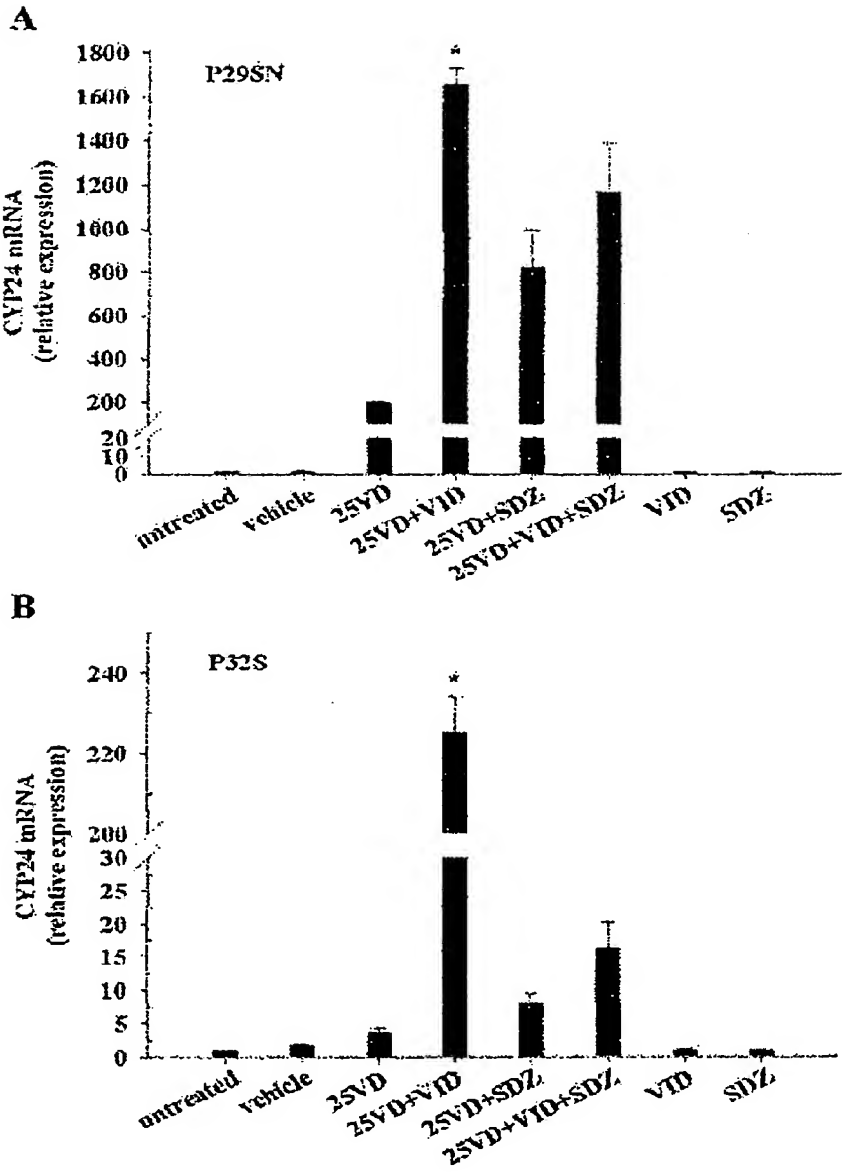

Figure 8. The effects of inhibitors for 1α-hydroxylase and 24-hydroxylase activities on the induction of 24-hydroxylase mRNA by 25OHD$_3$ in P29SN and P32S cells. P29SN (*A*) and P32S (*B*) cells grown to 70% confluence under standard conditions were incubated with vehicle (0.05% ethanol), 250 nM 25OHD$_3$ (25VD) individually or in the presence of VID400 at 100 nM (VID) or SDZ88-357 at 1000 nM (SDZ) for 6 h. 24-Hydroxylase (CYP24) mRNA was measured by quantitative real-time RT-PCR. Values are the mean ± SD of two independent experiments performed in duplicate. VID significantly increased mRNA level compared with 25VD alone in P29SN and P32S cells (*P<0.05).

Fig. 9

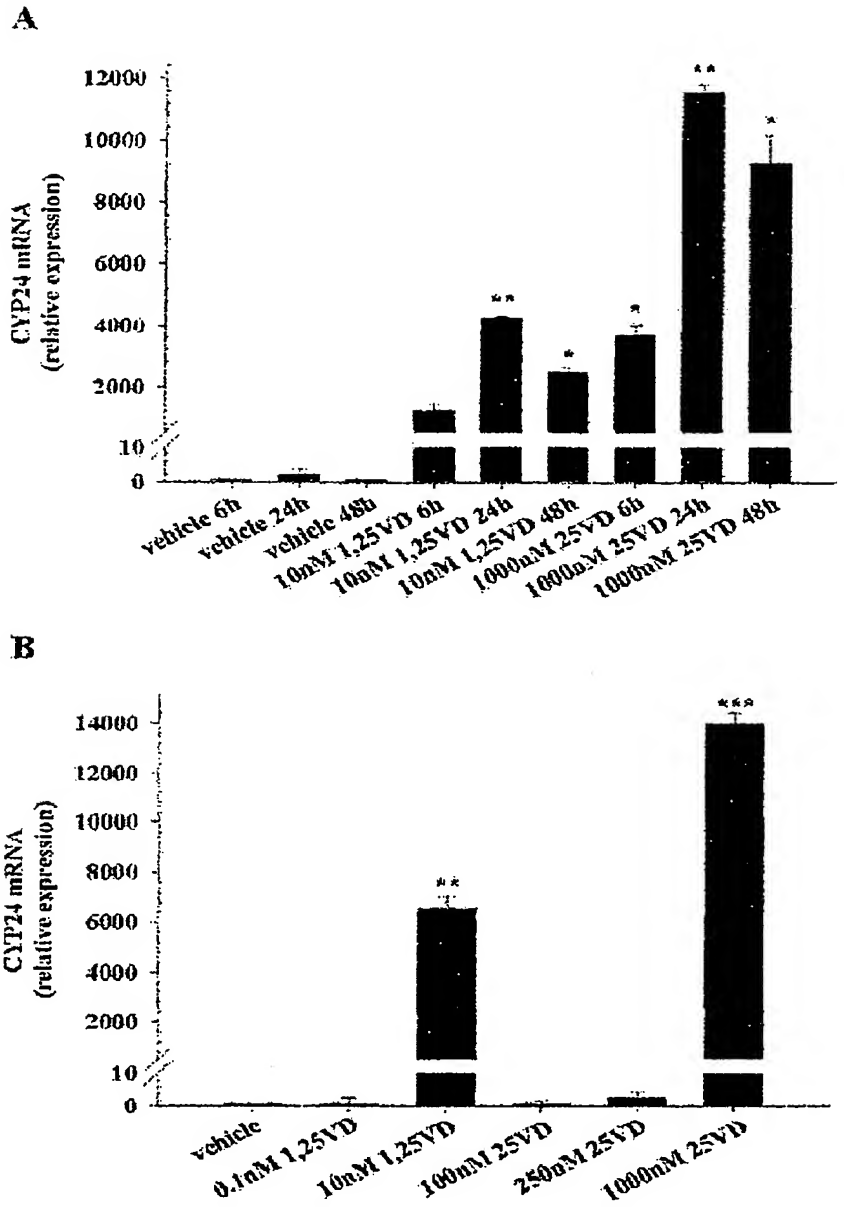

Figure 9. Induction of 24-hydroxylase mRNA by 1α,25-$(OH)_2D_3$ and 25OHD_3 in LNCaP cells. ***A***) LNCaP cells grown to 60% confluence in 10% DCC-FBS containing medium were incubated with vehicle (0.05% ethanol), 10 nM 1α,25-$(OH)_2D_3$ (1,25VD) or 1000 nM 25OHD_3 (25VD) for 6, 24, and 48 h. ***B***) LNCaP cells grown to 60% confluence in 10% DCC-FBS containing medium were incubated with vehicle (0.05% ethanol), 1α,25-$(OH)_2D_3$ (1,25VD) or 25OHD_3 (25VD) at the concentrations indicated for 24 h. 24-Hydroxylase (CYP24) mRNA was measured by quantitative real-time RT-PCR. Values are the mean ± SD of two (***A***) or three (***B***) independent experiments performed in duplicate.

Fig. 10

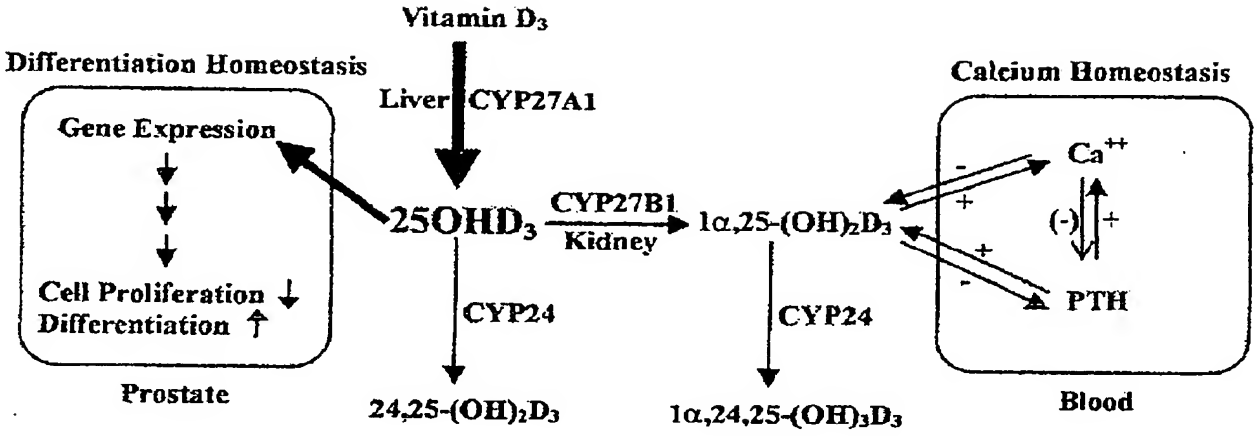

Figure 10. Diagram illustrating two vitamin D_3 endocrine systems. Under physiological conditions, the major circulating metabolite, $25OHD_3$, regulates gene expression in extrarenal tissues, for example, the prostatic stroma, resulting in the regulation of several vitamin D responsive genes involved in cell proliferation and differentiation. However, $1\alpha,25\text{-}(OH)_2D_3$ produced from $25OHD_3$ by 1α-hydroxylation in the kidney mediates calcium homeostasis by regulating serum calcium and parathyroid hormone. The sensitivities of differentiation homeostasis and calcium balance to $25OHD_3$ and $1\alpha,25\text{-}(OH)_2D_3$ are different. Ca^{++}, calcium; PTH, parathyroid hormone; CYP27A1, vitamin D_3 25-hydroxylase; $24,25\text{-}(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; $1\alpha,24,25\text{-}(OH)_3D_3$, $1\alpha,24,25$-trihydroxyvitamin D_3; +, up-regulation; –, down-regulation; (–), modest down-regulation.